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POLYPEPTIDES WHICH POSSESS AN ACTIVITY OF THE β-SECRETASE TYPE

TECHNICAL FIELD

The present invention relates to novel polypeptides and their pharmaceutical use. More particularly, the present invention relates to novel polypeptides which possess an activity of the β-secretase type and which are characterized in that they are able to cleave the natural β-amyloid peptide precursor (APP) specifically.

BACKGROUND OF THE INVENTION

Individuals who are suffering from Alzheimer's disease exhibit characteristic symptoms of memory distortion and loss of intellectual capacity and cognitive functions. These pathological changes are accompanied by neuronal atrophy, a substantial depletion in a particular type of receptor and also a reduction in synaptic connections. This syndrome involves the presence of a very substantial quantity of senile plaques and neurofibrillary degeneration, principally in the cerebral cortex, the hippocampus, the amygdaloid nucleus and the blood vessels of the cortex.

The so-called senile plaques are spherical structures which become slowly established over ten or so years in the extracellular spaces of the hippocampus, the cortex and other regions of the cerebrum. Their major

constituent is the β-amyloid peptide (βA), which is associated with other abnormal proteins. These structures are surrounded by abnormal axons and neurones.

The neurofibrillary degeneration is due to an accumulation of dense tracts of abnormal fibers in the cytoplasm of particular neurones, principally the pyramidal cells of the cortex. These neurofibrillary tangles consist of a particular form of the tau protein which, when associated with other proteins, gives rise to pairs of helicoidal neurofilaments which disrupt conduction of the nerve impulse.

Familial forms of this disease have been listed and appear to result from various genetic changes, all of which give rise to an abnormal accumulation of the βA peptide. These latter changes, which are very heterogeneous, have, in particular, been linked to various mutations on chromosomes 1, 14 and 21. This latter chromosome has aroused all the more interest because it carries the gene which encodes the βA precursor protein. It can be understood, therefore, why Alzheimer's disease appears at an early age (55) in individuals suffering from Down's syndrome (trisomy 21).

It is to be noted that individuals suffering from familial forms of the disease only constitute a low percentage of all those affected.

In almost all cases of Alzheimer's disease which are not linked to familial forms, those individuals who are more than 70 years old exhibit senile plaques in various regions of the brain. On the other hand, distribution of the plaques differs depending on the type of dementia concerned.

The human βA peptide, which has a molecular mass of 4 kDa, is generated by proteolytic cleavages of its precursor (APP) at the Met^{596}-Asp^{597} and

Val^{636}-Ile^{637} sites. The liberated molecule consists of 39 (to 42) amino acids having the following protein sequence:

DAEFRHDSGY[10] EVHHQKLVFF[20] AEDVGSNKGA[30] IIGLMVGGVV[40] IA (SEQ ID #1)

In aqueous solution, this peptide adopts a three-dimensional arrangement of the β-pleated sheet type. Its very hydrophobic COOH-terminal moiety confers on it aggregation properties, with the degree of oligomerization being a function of the pH (maximum formation at pH=5.4) and of the concentration of the peptide. In addition, the sequence between the Gly^{25} and Met^{35} residues confers neurotrophic and neurotoxic properties on this peptide.

The βA peptide is a natural product which is secreted by the cells and which can be detected in the blood and the cerebrospinal fluid. Although this peptide is neurotoxic, it is not, however, produced in sufficient quantities to form amyloid plaques. It is thought that an altered processing, or an overexpression, of its precursor predispose to the βA being deposited in the brain.

The primary transcript of the β-amyloid peptide precursor (APP) undergoes alternative splicing to generate mRNAs which encode at least 5 isoforms of 563, 695, 714, 751 and 770 amino acids (a.a.), which isoforms are expressed ubiquitously in the tissues, with their levels differing depending on the cell type.

However, the APP695 and 751 isoforms are exclusively restricted to the central and peripheral nervous system (in particular within the synapses in the astrocytes and the neurones), where they can play a role in the physiological

activity of the synapses. The APP751, APP563 and APP770 isoforms contain an insert of 56 a.a., which is homologous to the "Kunitz"-type protease inhibitor. Furthermore, the secreted form of APP 751 is identical to nexin II, which is a protease inhibitor which is involved in regulating extracellular serine proteases.

APP is a glycoprotein of approximately 120 kDa which exhibits the characteristics of a type II surface receptor. Although the true function of APP has still not been elucidated, studies have shown that this glycoprotein could play a role in regulating cell growth and also in adhesion interactions in inflammation, regeneration and the immune response.

All the APP isoforms are inserted into the endoplasmic reticulum owing to their signal sequence. The precursor is then targeted to the Golgi apparatus, where it undergoes various posttranslational modifications, after which it is anchored in the membrane. In the membrane, the APP can then, under the action of various proteases, undergo a variety of cleavages (see Figure 1), some of which are predominant:

The protease activity termed α-secretase activity cleaves in the interior of the βA sequence, between the Lys^{612} and Leu^{613} residues of APP695, in order to generate a secreted NH_2-terminal fragment (designated $sAPP_{\alpha}$:soluble APP_{α}), which contains the first 16 a.a. of βA.

The protease activity termed β-secretase activity cleaves the peptide bond of the Met^{596}-Asp^{597} doublet within the precursor in order to release a secreted NH_2-terminal APP fragment (designated $sAPP_{\beta}$:soluble APP_{β}), from which βA has been totally deleted.

The 3rd protease activity, termed γ-secretase activity, could also act between the Val^{636} to Ile^{637} residues of the precursor in order to generate a secreted proform, APP_{γ}, which contains βA.

The major constituent of the senile plaques which appear both in the familial forms and in the non-familial forms of Alzheimer's disease is the β-amyloid peptide (βA).

The βA peptide results from the cleavage of its precursor, i.e. APP, at the Met^{596}-Asp^{597} site of APP, in accordance with a protease activity of the β-secretase type, and at the Val^{636}-Ile^{637} site, in accordance with a protease activity of the γ-secretase type.

One mutation related to the β-secretase cleavage site has been identified among the familial forms forms of Alzheimer's disease. This mutation is the double "Swedish" mutation of APP (Lys^{595}-Met^{596} ⇒ Asn-Leu in APP695), which results in an increased production of the βA peptide (and therefore an increase in the maturation of APP in favor of the amyloidogenic pathway).

However, the fact still remains that, in the very great majority of cases of Alzheimer's disease, the APP is in its natural form with an unmutated β-secretase cleavage site.

Certain proteases derived from man, rats or monkeys have been studied by various authors and are assumed to be involved in the maturation of the APP precursor. Of these enzymes, those which may very particularly be mentioned are the serine proteases 1 and 2 (Abraham et al. (1991), Biochem. Biophys. Res. Commun., 174, 790-796; Matsumoto et al. (1994), Biochemistry, 33, 3941-3948;

Matsumoto et al. (1994), Neurosciences Letters, 195, 171-174) and the cathepsin G-like protease (Razzaboni et al. (1992), Brain Research, 589, 207-216). While these enzymes of human or simian origin cleave within the Met^{596}-Asp^{597} site of APP in accordance with a protease activity of the β-secretase type, they have been detected in, or partially purified from, patients suffering from Alzheimer's disease.

Given that the formation of the β-amyloid peptide results from the action of an enzyme of the β-secretase type on APP, it is easy to understand the importance of identifying and characterizing (an) enzyme system(s) of the β-secretase type which is/are selectively responsible for the post-translational maturation of the β-amyloid peptide precursor at the Met^{596}-Asp^{597} site in human cells which are not derived from patients suffering from Alzheimer's disease. Knowledge of these novel enzyme systems thus makes it possible to envisage preparing novel molecules which can be used pharmaceutically and which are, in particular, able to intervene in the metabolism of the β-amyloid peptide in non-familial forms of Alzheimer's disease.

SUMMARY OF THE INVENTION

The present invention provides the identification and characterization of polypeptides which possess a catalytic activity of the β-secretase type with regard to the β-amyloid peptide precursor (APP). Contrary to the other identified proteases, the polypeptides of the present invention have a specificity of action towards the natural form of APP. The present invention ensues, in particular, from demonstrating a 70 kDa polypeptide which is able to

cleave non-mutated forms of APP. The present invention also provides a process for enzymatically purifying the novel polypeptides, novel cell lines, and novel polyclonal and monoclonal antibodies directed against the polypeptides of the present invention. In addition, the present invention provides a process for identifying compounds which partially or completely inhibit the interaction of the novel polypeptides and the β-amyloid peptide precursor and/or to modulate or inhibit the β-secretase activity of the novel polypeptides. Novel pharmaceutical compositions are also provided which contain as the active ingredient, a polypeptide, an antibody or antisense nucleotide of the present invention.

Figure legends

Figure 1: Topography and cleavage sites of APP.

Figure 2: Description of the process for purifying the polypeptides of the invention.

Figure 3: Immunoblot analysis of the cleavage, by the polypeptides of the invention, of the complete "normal" (APP-K^{595}M^{596}) and "double mutated" (APP-N^{595}L^{596}) β-amyloid peptide precursors of membrane origin. Demonstration of the cleavage specificity of the polypeptides of the invention towards the "normal" (APP-KM) β-amyloid peptide precursor of membrane origin.

For each of the precursors (APP-NL and APP-KM), track 1 depicts the unincubated membranes without enzyme, track 2 depicts the membranes incubated at 37EC without enzyme, while track 3 corresponds to the membranes incubated at 37EC together with the polypeptides of the invention exhibiting an activity of the β-secretase type.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to polypeptides, or their variants, which possess an activity of the β-secretase type and which are characterized in that they are able to cleave the natural β-amyloid peptide precursor (APP) specifically.

Within the meaning of the present invention, the term "variant" denotes any molecule which possesses the same activity as the polypeptides of the invention and which is obtained by genetic and/or chemical modification of the peptide sequence. Genetic and/or chemical modification may be understood as meaning any mutation, substitution, deletion, addition and/or modification of one or more residues. Such variants can be generated for different purposes, such as that of improving its levels of production, that of increasing its resistance to proteases, that of increasing and/or modifying its activity, or that of conferring on it novel biological properties. Variants resulting from an addition which may, for example, be mentioned are chimeric polypeptides which contain an additional end-linked heterologous moiety. The term variant also comprises polypeptides which are homologous to the polypeptides described in the present invention and which are derived from other cell sources, in particular cells of other organisms.

The substrate which is cleaved by the polypeptides of the invention does not exhibit any mutation in its peptide sequence, and, in particular, the β-amyloid peptide precursor does not carry the double Swedish mutation. The polypeptides of the invention, or their variants, are able to selectively cleave the peptide bond of the Met^{596}-Asp^{597} doublet within the native or natural form of

APP. In particular, the polypeptides of the invention do not cleave the forms of APP which possess the Swedish mutation (Lys^{595}-Met^{596} ⇒ Asn-Leu), with this latter fact having been demonstrated on samples obtained from the brains of patients suffering from Alzheimer's disease.

The polypeptides according to the invention were purified from human cells from individuals who were not suffering from Alzheimer's disease and are able to cleave exclusively the natural form of APP within the peptide bond of the Met^{596}-Asp^{597} doublet.

The polypeptides of the invention are characterized in that their activity does not depend on a second substrate and/or a ligand. Examples of the latter which may be mentioned are ions, more specifically cations such as magnesium cations or calcium cations. Thus, other proteins, such as serine proteases 1 and 2 or cathepsin G-like protease, possessing a protease activity require the presence of calcium in order to be active.

The polypeptides according to the invention possess a molecular mass of between 65 and 75 kDa, and their molecular mass is preferably about 70 kDa. Their isoelectric point is between 6.0 and 7.0, and is preferably equal to 6.0.

These polypeptides are endopeptidases of the serine protease family. Preferably, these endopeptidases are of the chymotrypsin-sensitive type. Thus, the inhibition profile shows that these endopeptidases are totally inhibited by PMSF (phenylmethylsulfonyl fluoride), and partially inhibited by pefablock,

TPCK(L-1-chloro-3-[4tosylamido]-4-phenyl-2-butanone) and benzamidine. Furthermore, they are totally resistant to inhibition with antipapain.

The polypeptides of the invention, or their variants, are characterized by having a maximum β-secretase activity at a pH of between 7 and 8.

The invention also relates to non-peptide compounds, or compounds which are not exclusively peptide in nature, which compounds are able to cleave the β-amyloid peptide precursor at the Met^{596}-Asp^{597} site. Such compounds are obtained by duplicating the active motifs of the polypeptide according to the invention with non-peptide structures, or structures which are not exclusively peptide in nature, which are compatible with a pharmaceutical use. In this regard, the invention relates to the use of polypeptides as described above for preparing non-peptide molecules, or molecules which are not exclusively peptide in nature, which are active pharmacologically, by determining the structural elements of these polypeptides which are important for their activity and duplicating these elements with non-peptide structures or structures which are not exclusively peptide in nature. The invention also relates to pharmaceutical compositions which comprise one or more molecules which have been prepared in this way.

According to a variant of the invention, the polypeptides, or their variants, additionally comprise a signal sequence which enables them to be located precisely in the cell. Of the sequences which can be used, those which may be mentioned as being preferred are the sequence of the signal peptide of IgkB, the

signal peptide of APP, the signal peptides of the subunits of the muscle and central nervous system nicotinic acetylcholine receptors, etc.

The invention also relates to a process for enzymically purifying the polypeptides of the invention, which polypeptides possess an activity of the β-secretase type and are able to specifically cleave the natural precursor of APP. This process comprises the following steps:

- the supernatant from the cell culture is first of all concentrated on membranes.

- the concentration product then undergoes the various steps of the purification including, in particular, a step of tangential membrane centrifugation, followed by a step of exclusion chromatography and a step of ion exchange chromatography, then a step of hydrophobic interaction chromatography and, finally, a further step of exclusion chromatography.

The present invention also relates to the use of a cell line. This cell line was selected from a large number of other human cell lines (see Examples) which, while being of diverse origin, derive from individuals who are not suffering from Alzheimer's disease. These cell lines were used to look for polypeptides of the invention or their variants. Thus, these human cell lines represent the central or peripheral nervous system and the immune system and are able to carry out the normal metabolism of the β-amyloid peptide precursor which leads to the latter being produced. The cell line selected is preferably the monocyte-derived THP1 cell line (ATCC TIB 202).

The cell lines described above are used in particular as hosts for detecting compounds (ligands, antagonists or agonists) capable of inhibiting the interaction between the polypeptides of the invention and their substrate.

The invention also relates to polyclonal or monoclonal antibodies or antibody fragments which are directed against a polypeptide as defined above. These antibodies can be generated using methods known to the skilled person. In particular, these antibodies can be prepared by immunizing an animal against a polypeptide of the invention, or of one of its variants, and then withdrawing the blood and isolating the antibodies. These antibodies can also be generated by preparing hybridomas in accordance with the techniques known to the skilled person. The antibodies or antibody fragments according to the invention can be used, in particular, for their ability to at least partially inhibit the interaction between the said polypeptide and the β-amyloid peptide precursor and/or for at least partially inhibiting the β-secretase activity of the polypeptides of the invention with regard to the natural β-amyloid peptide precursor. In particular, these antibodies are used as medicinal products, especially for treating neurodegenerative diseases such as Alzheimer's disease.

The present invention also relates to a process for identifying compounds which are able to at least partially inhibit the interaction of the polypeptide and the β-amyloid peptide precursor and/or to at least partially modulate or inhibit the β-secretase activity of the polypeptides of the invention.

These compounds are detected and/or isolated in accordance with the following steps:

- a molecule or a mixture containing different molecules, which may not have been identified, is brought into contact with a recombinant cell such as expressing a polypeptide of the invention under conditions which would enable the said polypeptide and the said molecule to interact if the latter possessed an affinity for the said polypeptide, and

- the molecules which are bound to the said polypeptide of the invention are detected and/or isolated.

In a particular embodiment, this process of the invention is adapted to detecting and/or isolating agonists and antagonists of the β-secretase activity of the polypeptides of the invention. Based on these agonist or antagonist molecules, it is possible to use standard techniques known to the skilled person, in particular sequencing, to obtain their corresponding nucleotide sequences.

Thus, according to one variant of the invention, it can be particularly advantageous to express molecules which are agonists or antagonists of the polypeptides of the invention in situ from their nucleotide sequences. The preparation of these molecules, and their expression in vivo, ex-vivo, and/or in vitro, require their nucleotide sequences to be carried by a viral or plasmid vector and to be transfected, by means of the said vector, into appropriate host cells.

The present invention also relates to the use of the previously defined polypeptides, or their variants, for detecting ligands and compounds which are able to at least partially inhibit the interaction between the polypeptide and the β-amyloid peptide precursor and/or inhibit the β-secretase activity of the polypeptides of the invention or of their variants and/or intervene in the

metabolism of the natural β-amyloid peptide precursor and/or retard production of the β-amyloid peptide.

Thus, the present invention also relates to a method for detecting molecules which are able to influence the activity of the polypeptides of the invention.

This screening method comprises the following steps:

- the polypeptides of the invention, which exhibit an activity of the β-secretase type, are brought into contact with a molecule or a mixture which contains different molecules which may not have been identified.

- the reaction mixture described in the preceding step is brought into contact with the substrate of the polypeptides of the invention, which substrate is preferably APP in its natural form,

- the β-secretase activity on the APP is measured,

- the molecules which had an effect on the β-secretase activity of the polypeptides of the invention are detected and/or isolated.

The invention also relates to the use of a ligand or a modulator, which has been identified and/or obtained using the above-described process, as a medicament. Thus, such ligands or modulators can, by virtue of their ability to interfere with the β-secretase activity level of the polypeptides of the invention with regard to the natural β-amyloid peptide precursor, make it possible to treat certain neurological ailments, in particular Alzheimer's disease.

The invention also relates to any pharmaceutical composition which comprises, as the active principle, either a polypeptide as defined above or the previously defined agonist and antogonist molecules or ligands.

It also relates to any pharmaceutical composition which comprises, as the active principle, at least one antibody or one antibody fragment as defined above and/or an antisense oligonucleotide.

Furthermore, it also relates to the pharmaceutical compositions in which the above-defined peptides, antibodies, ligands and/or nucleotide sequences are combined with each other or with other active principles.

The pharmaceutical compositions according to the invention can be used for at least partially inhibiting the interaction of the polypeptides of the invention, or of their variants, with the natural β-amyloid peptide precursor and/or for at least partially inhibiting the β-secretase activity and/or intervening in the metabolism of the β-amyloid peptide precursor for the purpose of inhibiting or retarding production of the β-amyloid peptide. The pharmaceutical compositions are more preferably pharmaceutical compositions which are intended for treating neurodegenerative diseases such as Alzheimer's disease.

The present invention also relates to the use of the previously described molecules (ligands, antibodies or antibody fragments, antagonists and agonists) for at least partially inhibiting the interaction of the polypeptides of the invention, or of their variants, and the natural β-amyloid peptide precursor and/or for at least partially inhibiting the β-secretase activity of the polypeptides of the invention, or of their variants, and/or intervening in the metabolism of the β-amyloid peptide precursor for the purpose of inhibiting or retarding production of the β-amyloid peptide. The use of these molecules is preferably envisaged as a medicinal product, especially for treating neurodegenerative diseases and in particular for treating Alzheimer's disease.

According to one variant of the invention, the polypeptides of the invention, or their variants, are used for intervening in the metabolism of the β-amyloid peptide.

According to another embodiment of the invention, the above-defined polypeptides or their variants are used for detecting ligands or compounds which are able to at least partially inhibit the interaction between the polypeptides of the invention, or their variants, and the natural β-amyloid peptide precursor and/or for at least partially inhibiting the β-secretase activity of the polypeptides of the invention, or of their variants, and/or intervening in the metabolism of the β-amyloid peptide precursor for the purpose of inhibiting or retarding production of the β-amyloid peptide.

For their use according to the present invention, the polypeptides of the invention and, in particular, their antagonists, agonists, antibodies and ligands, are preferably combined with one or more excipients which is/are pharmaceutically acceptable for being formulated with a view to administration by the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, etc. route. They are preferably employed in an injectable form. The injectable forms can, in particular, be sterile, isotonic saline (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, etc., or mixtures of such salts) solutions, or dry, in particular lyophilized compositions which, by the addition of sterilized water or of physiological saline, as the case may be, enable injectable solutions to be constituted.

The present invention will be explained in more detail with the aid of the following examples, which are to be regarded as being descriptive and not limiting.

EXAMPLES

Origin of the cell lines

13 human cell lines of varied origin were used to look for maturation enzymes:

Central nervous system

SW 1088	ATCC HTB 12	Astrocytoma
SW 1788	ATCC HTB 13	Astrocytoma
U-138 MG	ATCC HTB 16	Glioblastoma
U-373 MG	ATCC HTB 17	Glioblastoma, astrocytoma, grade III

Peripheral nervous system

HMCB	ATCC CRL 9607	Bowes melanoma
Hs27	ATCC CRL 1634	Newborn foreskin
MRC5	ATCC CCL 171	Lung, diploid

Immune system

DAKIKI	ATCC TIB 206	B-cell, Ig A-secreting
H9	ATCC HTB 176	T-cell lymphoma
IM-9	ATCC CCL 159	Lymphoblast, Ig-secreting
K-562	ATCC CCl 243	Chronic myelogenous leukemia

RPMI 1788		ATCC CCL 156	Peripheral blood, IgM-secreting
THP1	ATCC TIB 202	Monocyte	

Cell culture

After thawing, the cells are cultured, depending on their origin, either in DMEM medium or in RPMI 1640 medium in the presence of 10% fetal calf serum. These cultures were carried out at 37°C in 1 liter flasks with the culture media being renewed every 2 to 3 days. Depending on the cell line studied, a period of from 2 to 5 months is required in order to obtain an 18 liter volume of culture medium. The last culture step is carried out over 48 hours in the absence of fetal calf serum and phenol red. These cell cultures are then centrifuged in order to recover the supernatant, which is used for purifying the enzymes.

The cell lines HMCB, U-373 MG, U-138 MG, MRC5 and Hs27 were cultured in DMEM medium while the cell lines SW 1088, SW 1783, K-562, H9, DAKIKI, THP1, RPMI 1788 and IM-9 were cultured in RPMI 1640 medium.

Enzyme purification

The 18 liters of supernatant from each cell culture are concentrated on ULTRASETTE™ (FILTRON) membranes having a cut-off threshold of 10 kDa, and the resulting concentration product was then used for purifying the proteolytic activities in accordance with the following protocol:

- The first step consists in centrifuging at 7000 rpm on a tangential membrane. More specifically, the concentration is effected on an ULTRAFREE7 (MILLIPORE) membrane having a cut-off threshold of 10 kDa

- A step of exclusion chromatography is then carried out. In accordance with one particular embodiment of the invention, the exclusion chromatography was carried out on a Sephacryl S-100 (Pharmacia) column whose exclusion limits are 10^3 Da and 10^5 Da.

- A step of ion exchange chromatography represents the third step of the process. For this, use was made, in particular, of a Q-Sepharose (Pharmacia) column whose gel consists of strong anions. The column is eluted with a 0 to 1 M saline gradient using solvent A (25 mM Tris, pH 7.5) and solvent B (25 mM Tris, 1 M NaCl, pH 7.5).

- The penultimate step consists of a step of hydrophobic interaction chromatography, in particular on a phenyl-Sepharose 6 (Pharmacia) column having a high degree of substitution (40 μmol/g of gel). This column was eluted with a gradient of from 1 to 0 M ammonium sulfate using solvent A (25 mM Tris, 1 M $(NH_4)_2SO_4$, pH 7.5) and solvent B (25 mM Tris, pH 7.5).

- Finally, the last step is a step of exclusion chromatography, which is carried out, in particular, on a TSKgel G2000SW (Interchim) column whose gel consists of rigid silica supports which are grafted with a hydrophilic group. The eluant is a 25 mM Tris buffer, pH 7.5, containing 250 mM NaCl.

Enzyme tests

The β-secretase activities were monitored by means of tests which used various peptides which mimicked or duplicated the amino acid sequence of the APP precursor at the level of the β-secretase-type enzyme cleavage site (Table 1).

In order to produce the chromophore peptide, 5 μl of peptide Z-Val-Lys-Met-MCA (7-amino-6-methylcoumarin), diluted 1/1000, are incubated with 5 μl of supernatant at 37EC for 6 hours. The reaction is stopped by adding 3 μl of 0.1N HCl and the enzyme activity is determined by measuring the fluorescence of the free AMC chromophore at 460 nm.

Synthetic peptides, which were of different sizes and which mimicked or duplicated the β-secretase-type enzyme cleavage site were synthesized in order to be used as substrates in studying the characterization and specificity of the enzymes (Table 1).

5 μl of supernatant are incubated with 5 μl of peptide at 37EC for 6 hours. The reaction is stopped by adding 3 μl of 0.1N HCl and the enzymic acvitity is determined by measuring the optical density, at 215 nm, of the cleavage fragments, which have been previously separated by HPLC.

The cleavage sites are deduced by determining the sequence of the fragments resulting from the cleavage.

The percent cleavage [%=100(A_0-A_j)/A_0] of each peptide substrate was evaluated by measuring the absorbance at 215 nm (A) of the substrate when incubated in the absence (A_0) and in the presence (A_j) of the enzyme under identical experimental conditions (incubation time, pH and concentration).

The percent inhibition [%=100(I_0-I_j)/I_0] of each substrate incubated in the presence of the enzyme was evaluated by measuring the absorbance at 215 nm, in the case of a peptide substrate, or the fluorescence at 460 nm, in the

case of the substrate Z-Val-Lys-Met-MCA, in the absence (I_0) and in the presence (I_j) of the inhibitor, under the same experimental conditions.

The normal APP precursor (APP-$K^{595}M^{596}$) and the APP precursor possessing the double "Swedish" mutation (APP-$N^{595}L^{596}$) were obtained from membrane extracts of insect cells which were infected with baculovirus containing the human genes encoding these precursors. These membrane extracts, which are incubated with the purified polypeptides of the invention possessing a β-secretase activity, are analyzed by immunoblotting using the antibody WO-2 (Ida N. et al. (1996) J. Biol Chem 271, 22908-22914), which is directed against the initial amino acids of the β-amyloid peptide, and the monoclonal antibody 22C11 (Boehringer Mannhein; Hilbich C. (1993) Journal of Biochemical Chemistry, 268, 26571-26577), which is directed against the NH_2-terminal motif of the precursor.

Example 1. Detecting the enzyme activities

The aim of this example is to detect enzyme activities in human cell lines from individuals who are not suffering from Alzheimer's disease.

Two approaches were used for detecting proteases likely to be involved in the maturation of human APP.

Immunological approach:

Taking the 13 human cell lines described in Materials and Methods as the starting material, the search for enzyme activities was carried out using the monoclonal antibody 22C11 for selecting the cell lines which had the ability to produce measurable quantities of APP at the level of the membrane and in the cell

culture medium. The monoclonal antibody WO-2 was used to reveal and identify the various sites at which the APP was cleaved.

The following results were obtained:

- Use of the monoclonal antibody 22C11 enabled 8 cell lines (HMCB, MRC5, Hs27, SW1088, SW1783, H9, THP1 and IM-9) to be selected out of the total of 13 tested. In the case of the cells which were selected, immunoblot analysis also demonstrated a difference in molecular mass between the membrane APP (120 kDa) and the soluble APPs (110-100 kDa). This indicates that the COOH-terminal sequence of the precursor underwent one or more enzyme cleavage(s).

- immunoblot analysis, using the monoclonal antibody WO-2, of the molecular entities generated in the 8 cell lines selected made it possible to reveal and identify the different sites at which the APP was cleaved and to show that the βA peptide precursor underwent differential maturation.

Thus, this approach made it possible to select cell lines which have the ability to produce measurable quantities of APP at the level of the membrane and in the cell culture medium and to demonstrate enzyme cleavages in the βA peptide precursor.

Peptide substrates:

The peptides [KMD]APP(-5,+5) and Z-Val-Lys-Met-MCA which are derived from APP and which mimic the cleavage site, were used as substrates for detecting the different enzyme activities present in the 8 cell lines.

A combined analysis (HPLC, amino acid composition and sequence determination) of the fragments generated by cleaving the substrate [KMD]APP(-5,+5) was carried out on the cell lines selected. Thus, after incubating the substrate [KMD]APP(-5,+5) with the supernatants, the fragments which were generated were first of all separated by HPLC on a reverse-phase RPC_{18} (VYDAC) column, which was eluted with a 5-40% acetonetrile/0.05% TFA gradient. The sequences and/or the amino acid compositions of these fragments were determined using standard techniques.

The results of this analysis made it possible to identify a predominant cleavage of the $\underline{Met^{-1}\text{-}Asp^{+1}}$ peptide bond (corresponding to the $Met^{596}\text{-}Asp^{597}$ site in the intact APP) and 2 minor cleavages of the $Ser^{-5}\text{-}Glu^{-4}$ bond (corresponding to the $Ser^{592}\text{-}Glu^{593}$ site in the intact APP) and the $Ala^{+2}\text{-}Glu^{+3}$ bond (corresponding to the $Ala^{598}\text{-}Glu^{599}$ site in the intact APP) in each of the 8 cell lines selected.

The inhibition profiles of the enzyme activities of the 8 cell lines with regard to the fluorescent substrate Z-Val-Lys-Met-MCA were also analyzed (Table 2).

The results of this latter analysis revealed the existence of major enzyme activities of the serine (inhibition by aprotinin and pefabloc) and metalloprotease (inhibition by EDTA and phosphoramidon) type in each of the 8 cell lines selected (Table 2).

Example 2. Purification and characterization of the β-secretase activity

The aim of this example is to describe the purification of the polypeptides of the invention possessing a β-secretase activity and to demonstrate their characteristics.

Based on the selection of the 8 human cell lines and the results obtained in Example 1, the cell line THP-1 was chosen, on account of its rapid cell cycle, making it possible to obtain large quantities of protein, for use as a model for purifying the sought-after β-secretase activity in accordance with the purification protocol described in "Materials and Methods".

An analysis of the residual activity of the fractions exhibiting proteolytic activities with regard to the substrates Z-Val-Lys-Met-MCA and [KMD]APP(-5,+5) was carried out with the aim of continuing the purification of the polypeptides of the invention. At each purification step, the different fractions were first of all brought into contact with the peptide Z-Val-Lys-Met-MCA in order to isolate the fractions which exhibited endoproteolytic activities. These latter fractions were then tested with regard to the [KMD]APP(-5,+5) peptide in order to isolate those fractions which preferentially cleave this peptide substrate at the Met^{-1}-Asp^{+1} peptide bond (corresponding to the Met^{596}-Asp^{597} site in the intact APP). The results of these studies made it possible to identify different fractions which exhibited endoproteolytic activities and which were isolated from supernatants of the 8 cell cultures selected using these two substrates in parallel.

Several protein fractions were obtained during the last step of the purification process, which is a step of exclusion chromatography on a TSK 2000 column.

Measuring the residual activity of these fractions with regard to the [KMD]APP(-5,+5) peptide made it possible to obtain a single fraction which has an activity of the β-secretase type. This fraction was characterized by measuring its molecular weight by polyacrylamide gel electrophoresis, measuring its isoelectric point, determining its maximum activity as a function of pH, and also determining its profile of inhibition by standard inhibitors ("Materials and Methods").

The electrophoresis analysis was carried out on a Phast-system (Pharmacia) 4-20% polyacrylamide gel under denaturing or normal conditions, and shows a band having a molecular mass in the vicinity of 70 kDa.

The maximum activity with regard to the [KMD]APP(-5,+5) peptide was observed at pH values of between 7 and 8.

The inhibition profile of this fraction with regard to the [KMD]APP(-5,+5) peptide shows that the fraction is a serine protease, with the calculated inhibition percentages being, respectively, 100% in the case of PMSF, 75% in the case of pefablock, 25% in the case of TPCK, 10% in the case of benzamidine and 0% in the case of antipapain.

This example therefore describes the purification process and the search for, and demonstration of, the different characteristics of the polypeptides of the invention possessing a β-secretase activity.

Example 3. Specificity of the β-secretase activity

This example describes the analysis of the β-secretase activity of the polypeptides of the invention.

This specificity was analyzed using different substrates such as:

- peptides which mimic or duplicate the amino acid sequence of the precursor at the level of the cleavage site and which are described in Table 1.

- the β-amyloid peptide precursor in its natural and mutated (Swedish mutation) forms.

The polypeptides of the invention were brought into contact with the different substrates and the percentage cleavage of these substrates was calculated. The results are presented in Table 3.

In the case of the synthetic peptides, analysis of the findings, which are compiled in Table 3, shows the characteristics relating to the importance of some subsites involved in the recognition of the substrate by this β-secretase and allow it to be concluded that:

1) The subsites P_1 and P_2 are essential (Part A of Table 3), with this being the case whatever the size of the substrates. It was observed that the double mutation (Lys-Met⇒Asn-Leu) totally abolishes the enzymic cleavage.

2) The subsites P_2 and P_1 are interactive or cooperative (Part B of Table 3). Thus, a single substitution in P_2 (Lys⇒Asn) or in P_1 (Met⇒Leu) only decreases the level of cleavage whereas the so-called "Swedish" double mutation abolishes recognition of the substrate.

Substitution of the residue in P_2 (Lys⇒Arg) finds expression in a difference between the levels of cleavage of the peptides having Leu in P_1 ([K<u>L</u>D]-APP(-5,+5) and [R<u>L</u>D]-APP(-5,+5)) which is greater than that observed in the case of substrates having Met in P_1 ([K<u>M</u>D]-APP(-5,+5) and [R<u>M</u>D]-APP(-5,+5)).

3) The size and/or the volume of the residue in P_1 are important (Part C Tab. 3):

The level of enzymic cleavage decreases when the constraint exercised on the peptide skeleton by the side chain of the P_1 subsite increases. Thus, the experiments which were carried out make it possible to obtain a grading of the level of cleavage in terms of the substitution effected:

[K<u>M</u>D]-APP(-5,+5)>[K<u>L</u>D]-APP(-5,+5)>[K<u>I</u>D]-APP(-5,+5)> [K<u>V</u>D]-APP(-5,+5)

4) The residue of the P'_1 subsite is necessarily Asp or Glu (Part D Tab. 3):

Thus, the results demonstrated that the mutation of Asp with Asn or Gln does not abolish cleavage of the substrate; what is more, the cleavage takes place at the <u>Ala-Glu</u> site, equivalent to the <u>Met-Asp</u> site; furthermore, the <u>Ala-Glu</u> pseudosite is only accessible in the natural substrate since, under the same experimental conditions, the level of cleavage of the APP(1,5) fragment is only 35%.

Thus, based on the results which were previously obtained with the polypeptides of the invention with regard to the β-secretase-type cleavage specificity, it is possible to envisage obtaining inhibitors which are of peptide,

pseudopeptide or non-peptide nature and which are competitive with the Met-Asp cleavage site.

In the case of the β-amyloid peptide precursors of membrane origin, the products of the cleavage of the "normal" (APP-$K^{595}M^{596}$) and "double mutated" (APP-$N^{595}L^{596}$) full-length precursors by the polypeptides, whose β-secretase activity was demonstrated in Examples 1 and 2, were visualized by immunoblotting using the antibodies 22C11 and WO-2.

Analysis of the molecules using these antibodies shows that the percentage cleavage of the APP-KM precursor increases whereas that of the APP-NL precursor remains virtually zero, with this being the case whatever the time of incubation in contact with the enzyme. Thus, the results presented in Figure 3 demonstrate that:

· in the case of the bac.NL membranes, that is to say the incubated membranes containing the APP-NL precursor, the same bands are found whatever the experimental conditions (lanes 1, 2 and 3). Thus, no cleavage by the polypeptides of the invention is observed.

· in the case of the bac.WT membranes, that is to say the incubated membranes containing the natural APP-KM precursor, a new band of about 12 kDa appeared in lane 3 as compared with the other two lanes. Lane 1 depicts the unincubated membranes without enzyme, lane 2 depicts the membranes which were incubated at 37EC without enzyme, while lane 3 corresponds to the membranes which were incubated at 37EC together with the polypeptides of the invention exhibiting an activity of the β-secretase type. It is to be noted that the

difference in the intensity of the bands between lanes 1 and 2 in the case of the bac.WT membranes is due to the quantity of starting material which was loaded onto the gel.

The analysis using the WO-2 antibody revealed this new band, which has a molecular mass of about 12 kDa and which corresponds to the COOH-terminal fragment derived from the precursor being cleaved by the β-secretase at the Met-Asp bond. This analysis permits the conclusion that the natural APP-KM precursor is cleaved by the polypeptides of the invention.

This result indicates, therefore, that the APP-KM precursor, and not the APP-NL precursor, was cleaved selectively, and confirms the findings obtained with the APP substrate peptides of 10, 20 or 40 amino acids in length.

In addition, this example demonstrates that the previously isolated polypeptides of the invention have a β-secretase-type activity which is specific for the natural β-amyloid peptide precursor.

Peptides	Amino acid sequences				
		P_2	P_1	P'_1	
APP(1,+5)				Asp	AEFR
[KMD]-APP(-5,+5)	SEV	Lys	Met	Asp	AEFR
[RMD]-APP(-5,+5)	SEV	<u>*Arg*</u>	Met	Asp	AEFR
[KLD]-APP(-5,+5)	SEV	Lys	<u>*Leu*</u>	Asp	AEFR
[RLD]-APP(-5,+5)	SEV	<u>*Arg*</u>	<u>*Leu*</u>	Asp	AEFR
[NLD]-APP(-5,+5)	SEV	<u>*Asn*</u>	<u>*Leu*</u>	Asp	AEFR
[NMD]-APP(-5,+5)	SEV	<u>*Asn*</u>	Met	Asp	AEFR
[KID]-APP(-5,+5)	SEV	Lys	<u>*Ile*</u>	Asp	AEFR
[KVD]-APP(-5,+5)	SEV	Lys	<u>*Val*</u>	Asp	AEFR
[KMN]-APP(-5,+5)	SEV	Lys	Met	<u>*Asn*</u>	AEFR
[KMQ]-APP(-5,+5)	SEV	Lys	Met	<u>*Gln*</u>	AEFR
[KM]-APP(-10,+10)	KTEEISEV	Lys	Met	Asp	AEFRHDSGY
[NL]-APP(-10,+10)	KTEEISEV	<u>*Asn*</u>	<u>*Leu*</u>	Asp	AEFRHDSGY
[KL]-APP(-10,+10)	KTEEISEV	Lys	<u>*Leu*</u>	Asp	AEFRHDSGY
[NM]-APP(-10,+10)	KTEEISEV	<u>*Asn*</u>	Met	Asp	AEFRHDSGY
[KM]-APP(-20,+20)	TRPGSLTNIKTEEISEV	Lys	Met	Asp	AEFRHDSGYEVHHQKLVFF
[NL]-APP(-20,+20)	TRPGSLTNIKTEEISEV	<u>*Asn*</u>	<u>*Leu*</u>	Asp	AEFRHDSGYEVHHQKLVFF

Table 1: Table of the peptides of different sizes which mimic or duplicate the β-secretase cleavage site and which were synthesized for being used as substrates in characterizing, and determining the enzyme specificity of, the polypeptide according to the invention.

Inhibitors	Cell lines							
	HMCB	**H9**	**Hs27**	**IM-9**	**MRC-5**	**THP-1**	**SW1088**	**SW1783**
Standard	100	100	100	100	100	100	100	100
E64 (0.1 mM)	97	87	88	100	87	82	100	92
EDTA (3.3 mM)	**43**	**59**	**27**	*71*	**31**	**54**	**39**	92
pepstatin (10 μM)	100	100	88	100	99	100	100	100
chymostatin (5 μM)	95	87	*72*	97	*75*	*71*	100	100
aprotinin (0.8 μM)	90	100	**40**	97	97	93	100	100
pefabloc (3.3 μM)	**53**	**62**	**13**	97	**34**	**54**	91	**54**
phosphoramidon (70 μM)	97	**62**	83	79	**66**	89	*70*	*76*
captopril (60 μM)	96	87	97	100	100	100	100	100

Tabl 2: Profile of inhibition of the enzymatic activities of the 8 cell lines selected with respect to the peptide **Z-Val-Lys-Met-MCA**, expressed as a percentage of activity.

P ptides	Cleavage (%)	Bond cleaved
(A)-Swedish mutation: effect of size		
[**KMD**]-APP(-5,+5)	**65**	**Met▼Asp**
[**NLD**]-APP(-5,+5)	**0**	
[**KM**]-APP(-10,+10)	**45**	**not determined**
[**NL**]-APP(-10,+10)	**0**	
[**KM**]-APP(-20,+20)	**90**	**not determined**
[**NL**]-APP(-20,+20)	**0**	
(B)-Swedish mutation: importance of the P_2 and P_1 subsites		
[**NMD**]-APP(-5,+5)	**45**	**Met▼Asp**
[**KMD**]-APP(-5,+5)	**65**	**Met▼Asp**
[**KLD**]-APP(-5,+5)	**60**	**Leu▼Asp**
[**NLD**]-APP(-5,+5)	**0**	
[**KMD**]-APP(-5,+5)	**65**	**Met▼Asp**
[**RMD**]-APP(-5,+5)	**80**	**Met▼Asp**
[**KLD**]-APP(-6,+5)	**60**	**Leu▼Asp**
[**RLD**]-APP(-5,+5)	**20**	**Leu▼Asp**
(C)-Substitution in P_1		
[**KMD**]-APP(-5,+5)	**65**	**Met▼Asp**
[**KLD**]-APP(-5,+5)	**60**	**Leu▼Asp**
[**KID**]-APP(-5,+5)	**40**	**Ile▼Asp**
[**KVD**]-APP(-5,+5)	**15**	**Val▼Asp**
(D)-Substitution in P'_1		
[**KMD**]-APP(-5,+5)	**65**	**Met▼Asp**
[**KMN**]-APP(-5,+5)	**70**	**Ala▼Glu**
[**KMQ**]-APP(-5,+5)	**80**	**Ala▼Glu**
APP(1,+5)	**35**	**Ala▼Glu**

Table 3: Results of the analysis of the enzyme specificity of the polypeptide of the invention using

peptides which mimic or duplicate the sequence of the amino acids of the APP precursor at the level of the cleavage site.

References

1)- Nelson et al. (1993), **Journal of neurochemistry, 61**, 567-577

2)- Sahasrabuche et al. (1993), **Journal of Biological Chemistry, 268** 16699-16704

3)- Higaki et al. (1996), **Journal of Biological Chemistry, 271**, 31885-31893

4)- Abraham et al. (1991), **Biochem. Biophys. Res. Commun., 174**, 790-796.

5)- Matsumoto et al. (1994), **Biochemistry, 33**, 3941-3948.

6)- Matsumoto et al. (1994), **Neurosciences Letters, 195**, 171-174.

7)- Razzaboni et al. (1992), **Brain Research, 589**, 207-216.

8)- LePage et al. (1995), **FEBS Letters, 377**, 267-270.

9)- Itoh et al. (1997), **Journal of Biological Chemistry, 272**, 22389-22392

10)- Papastoisis et al., (1994), **Biochemistry, 33**, 192-199.

11)- Thompson et al., (1995), **Biochem. Biophys. Res. Commun., 213**, 66-73

12)- Schönlein et al., (1994), **Biochem. Biophys. Res. Commun., 201**, 45-53

13)- Ida N. et al. (1996), **J. Biol Chem 271**, 22908-22914 "Analysis of heterogeneous βA4 peptides in human cerebrodpinal fluid and blood by a newly-developed sensitive Western blot assay".